Expression of breast cancer resistant protein and P-glycoprotein in cultured primary bovine mammary epithelial cells isolated from tissue and milk

Lerica Le Roux-Pullen (l.leroux@uu.nl)
Utrecht University
Vienna Van de Laarschot
Radboud University Nijmegen Medical Centre
Marjolein Oosterveer-van der Doelen
Utrecht University
Ronette Gehring
Utrecht University

Short Report

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Abstract

A tight monolayer of mammary epithelial cells, in which the transporter expression is well characterised, could be a useful in vitro model for studying how chemicals cross the blood-milk-barrier (BMB) and accumulate in milk. The efflux transporter breast cancer resistance protein (BCRP) is the predominant efflux transporter in the BMB. In order for an in vitro cell culture model to mimic the in vivo BMB, the expression of BCRP should be higher than that of the efflux transporter p-glycoprotein (P-gp).

The aim of the study reported in this communication was to determine the relationship between the transcription of ABCG2 and ABCB1 (encoding for BCRP and P-gp respectively) in primary bovine mammary epithelial cells in culture. Cells were sourced from both lactating udder tissue after routine slaughter of the donor, and then cryopreserved until use, as well as from fresh, raw milk of healthy cows in mid-lactation.

In this study a total of 18 RNA samples from 18 individual trypsinisations, originating from 6 individual isolations (3 milk isolations and 3 udder tissue isolations) were analysed by rt-PCR. In all eighteen samples analysed, the mRNA detected for P-gp was higher per sample than the mRNA detected for BCRP. In six samples no mRNA for BCRP was detected at 40 amplification cycles.

Our study showed that when pbMEC is cultured in the proliferation medium as described, P-gp mRNA expression is consistently higher than BCRP mRNA expression. This relationship is the inverse of the relationship between BCRP and P-gp in the BMB in vivo.

Introduction

During lactation a tight blood-milk barrier (BMB) is formed by mammary epithelial cells (MECs) connected by tight junctions. These junctions prevent the paracellular flow of compounds, allowing the BMB to regulate milk composition (Nguen, 1998). The BMB therefore plays an important role in determining the quality and safety of milk.

There are still a lot of unanswered questions regarding the cellular mechanisms by which chemical compounds are transported across the mammary epithelium. In vitro cell culture models are useful to study how chemicals cross epithelial barriers such as the BMB. To mimic the in vivo barrier, the epithelial cells in such a model should form a confluent monolayer to present a tight barrier against the paracellular flow of compounds. These cells must also be well characterised with regards to drug transporter expression and activity, to ensure accurate, reliable interpretation of data.

Endogenous and exogenous compounds can cross the BMB via the transcellular route by passive or facilitated diffusion, or active transport. Breast cancer resistance protein (BCRP, decoded by ABCG2), is a transporter of the ATP-binding cassette family that plays an important role in the active accumulation of substrates in milk (Jonker et al. 2005). BCRP is strongly induced (i.e. upregulated) in the lactating mammary gland of humans, mice and dairy animals when compared to non-lactating tissue (Jonker et

al. 2005; Lindner et al. 2013). In contrast, another important efflux transporter in mammals, multi-drug resistance protein, MDR1 (also known as P-glycoprotein, or P-gp, decoded as ABCB1), is downregulated in the mammary gland during lactation (Alcorn et al. 2002; Yagdiran, 2015). Both BCRP and P-gp have a broad range of substrates, that overlaps to some extent (Mao and Unadkat, 2015). The reason for this relative up- and downregulation of BCRP and P-gp respectively during lactation, remains unclear, but an important and consistent feature of this epithelial barrier in the lactating mammary gland is that BCRP is the predominant efflux transporter (Lidner *et al.*2013, Yagdiran, 2015).

An immortalised bovine mammary epithelial cell line frequently used by researchers studying the BMB is BME-UV (Zavision *et al.*1996). Several apical cationic and anionic transporters have been identified (Al-bataineh *et al*. 2009), but researchers have not been able to differentiate BME-UV cells to express P-gp and BCRP in a relationship that is representative of the lactating BMB. BCRP expression is low in this cell line (Yagdiran, 2015). To the authors' knowledge there are no mammary epithelial cell lines from dairy animals that express BCRP appropriately. Transfected cell lines, for example the MDCKII-BCRP cell line, have been used as an alternative by researchers to specifically study the transport of BCRP-substrates (Wasserman *et al*. 2013), but is less preferred as a biologically relevant option for the BMB, since these cells does not share all the characteristics of MECs. Primary bovine mammary epithelial cells (pbMEC) could have several advantages over immortalized and transfected cell lines. Primary cells are isolated from the tissue of interest, can be cultured directly and are not genetically manipulated. They are thus expected to have more normal cell morphology and maintain many of the important markers, responses and functions seen in vivo. The data obtained from primary cell culture experiments could be more relevant and representative of the in vivo environment and include individual donor characteristics (Mather and Barnes, 1998). Since first described in 1983 (McGrath), pbMECs are most commonly harvested from udder tissue at the slaughterhouse, with some variations in methodology between researchers. These pbMECs isolated from tissue requires several trypsinisations to purify the culture from co-isolated fibroblasts. An alternative method is to isolate cells from raw milk. This method as described by Beuhring (1990) was later refined by researchers such as Sorg (2012) and Danowski (2013). Both methods of pbMEC isolation have their own advantages and disadvantages. Important to note is that milk isolated cells can be used from the first passage as fibroblast contamination is no concern, but the number of cells isolated varies greatly between isolation attempts and there is also a high risk of contamination (Beuhring, 1990).

In a study by Vachkova and colleagues (2021), pbMEC isolated from udder tissue showed the presence of moderate quantities of BCRP mRNA at 80% confluence in culture. Their study concluded that this model should be further validated as a potentially interesting in vitro model for studying active transport across the BMB. Although this study investigated the expression and function of several biotransformation enzymes, as well as the transcription of BCRP, they did not consider P-gp. The aim of the study reported in this communication was to determine the relationship between the transcription of ABCG2 and ABCB1 (encoding for BCRP and P-gp respectively) in pbMECs in culture, at 80% and 100% confluence.

Materials And Methods

Two sources of pbMEC were used: (i) pbMECs isolated from lactating udder tissue from cows sent for routine slaughter at the slaughterhouse and then cryopreserved at the third or fourth passage; cryopreservation was necessary to preserve the cells until use, (ii) pbMEC isolated from fresh, raw milk of healthy cows in mid-lactation; milk was readily available and isolation could be done timely as needed for an experiment. For both isolations previously published methods were used (Le Roux-Pullen, 2015 - Master Thesis, University of Pretoria, Paraclinical Sciences Department; Danowski, 2013). Both sources of isolated cells were cultured in T25 culture flasks. All pbMECs were cultured in DMEM/F12 Ham medium (Gibco®), without Phenol-red, containing 10% foetal bovine serum (Biowest®), 100x insulin-transferrin-sodium selenite (Sigma-Aldrich), 1% penicillin-streptomycin, amphotericin B and gentamicin (Sigma-Aldrich), and L-glutamine (Gibco®), human epithelial growth factor (Sigma-Aldrich), and hydrocortisone (Sigma-Aldrich). The addition of growth hormones to the culture medium were different from the hormone-free culture medium of Vachkova and colleagues; preliminary work in our laboratory showed that pbMEC requires both hydrocortisone and epidermal growth factor to be able to grow to confluent monolayers within 2 weeks after seeding. Preliminary work in our laboratory showed that antibiotics and antimycotics are necessary to prevent contamination in cultures from milk isolated cells. The presence of pbMEC in culture was confirmed microscopically based on uniformity, with the characteristic cobblestone growth of epithelial cells and further confirmed through appropriate barrier formation on hanging inserts.

The expression of mRNA encoding for BCRP and P-gp were analysed with rt-PCR in four different cell types:

1. pbMECt-80% confluence: pbMEC isolated from the tissue of three donors, cryopreserved, and then again cultured to 80% confluence, at which time the cells were trypsinised with warm trypsin and a sample of the cell suspension, after centrifugation, preserved in Tri-reagent® and stored at -20°C until analysis. Cells from eight individual trypsinisations were analysed.
2. pbMECt-100% confluence: pbMEC isolated from the tissue of the three donors, cryopreserved, and then cultured to 80% confluence, at which point it was trypsinised and seeded on 6-well hanging inserts (ThinCert®), typically used for transport/efflux studies. These cells were in culture for at least 2 weeks until confluent monolayers with stable transepithelial resistance (TEER) measurements were formed. Good barrier integrity at this point was confirmed by a more than 96% rejection of the fluorescent dye Lucifer Yellow. Cells from four individual trypsinisations were analysed.
3. pbMECt-TR: Trypsin-resistant pbMEC selected from tissue-isolated, cryopreserved cells. The potential superiority of using trypsin-resistant MECs in primary cell culture was discussed by Kimura and colleagues (2006). For trypsin-resistant cells only the pbMECs remaining attached after a warm trypsinisation treatment is kept for further culturing to confluence. Cells from three individual trypsinisations were sub-cultured as TR-cells and subsequently analysed.

4. pbMECm-80% confluence: pbMEC isolated from milk and cultured to 80% confluence, at which time the cells were trypsinised with warm trypsin and a sample of the cell suspension, after centrifugation, preserved in Tri-reagent® and stored at -20°C until analysis. Cells from three donors and three individual trypsinisations were analysed.

Organic extraction of total RNA was done from the stored samples using chloroform and isopropanol as solvents, similar to Santos and colleagues (2021). The extracted RNA was then transcribed into complementary DNA by reverse transcriptase using a cDNA Synthesis Kit (BioRad®). The cDNA was finally used as the template for the qPCR reaction. SYBR Green was added to the PCR mixture to aid in detection and quantification of the PCR product. Forty cycles were used per reaction at a temperature of 64°C.

The primers used for BCRP and P-gp respectively was:

- CGCAGAAGGAGATGTGTT forward primer BCRP
- TTGGATCTTTCCTTGCTGCT reverse primer BCRP
- ATTTGGCAAAGCTGGAGAGA forward primer MDR1
- ACCCTGTAGCCCCTTTCACT reverse primer MDR1

A total of 18 RNA samples, from 18 individual trypsinisations, were analysed by rt-PCR. The cells cultured originated from 6 individual donors, 3 milk isolations and 3 udder tissue isolations.

Normalisation of the data was not done as the purpose of this study was to determine the relationship between the expression of mRNA encoding for BCRP and P-gp per individual sample, and not to compare expression between samples.

Results And Discussion

To mimic the in vivo situation during lactation, BCRP expression must be consistently higher than P-gp expression in pbMECs (Alcorn et al. 2002; Jonker et al. 2005; Lindner et al. 2013; Yagdiran, 2015). In this study quantification cycles (Cqs) served as a semi-quantitative value to rank the relative concentration of genetic material per sample for the mRNA of BCRP and P-gp. In rt-PCR Cqs levels are inversely proportional to the amount of target nucleic acid in a sample, i.e. the lower the Cq value, the higher the amount of specific mRNA present in the sample.

In all eighteen samples analysed, the mRNA detected for P-gp was higher than the mRNA detected for BCRP in a particular sample, demonstrated by the consistently lower Cq value of the P-gp mRNA (Fig. 1). In six samples no mRNA for BCRP was detected at the typical cut-off point of 40 amplification cycles: two out of the four samples from pbMEC on hanging inserts (after 2 weeks in culture, with good barrier formation), in two of the three milk-isolated pbMEC samples and in two of the eight pbMEC tissue samples at 80% confluence. These samples' BCRP Cq values are not included in Fig. 1 (as seen by the un-coupled squares of the associated P-gp values). In fourteen of the samples a very strong positive rt-PCR

reaction was seen for P-gp mRNA, characterised by a Cq value of less than 29 (Bustin et al. 2009). The trypsin-resistant pbMECs had the highest mRNA expression for P-gp in our study. In no sample a strong positive rt-PCR reaction was seen for BCRP mRNA.

The results of this study confirms previous unpublished work from our cell culture laboratory, where pbMEC isolated from fresh, raw milk, and cultured on hanging inserts for transport studies to a point of 99% rejection of Lucifer Yellow, had lower, to undetectable, mRNA expression for BCRP, compared to a higher mRNA expression for P-gp, with 40 amplification cycles. Vachkova and colleagues also cultured pbMEC to 80% confluence, but tight 100% confluent monolayers are a pre-requisite for a cell culture model to be used for studying active transport across the BMB. It is known that culture conditions and time in culture (as presented by the level of confluence in our study) can affect transporter expression of cells; specifically, the upregulation of P-gp in culture, with the corresponding downregulation of BCRP, has previously been described (Bark et al. 2008).

Our study showed that for pbMEC isolated from udder tissue and then cryopreserved, as well as for pbMEC isolated from fresh, raw milk, when cultured in the proliferation medium as described in this communication, at both 80% and 100% confluence, mRNA expression of P-gp is consistently higher than that of BCRP. To determine whether this relationship in mRNA levels of pbMEC in culture corresponds to protein expression levels, Western blot or targeted proteomics needs to be performed.

Conclusion

Culturing pbMEC as monolayers in vitro, in typical proliferation/growth medium, on a plastic surface, or hanging insert, for a timeframe up to 80% and 100% confluence of the cells, consistently results in a lower mRNA expression of BCRP than P-gp. This relationship in expression is the inverse of BCRP and P-gp expression seen in the lactating bovine mammary gland in vivo.

There is still a need for an improved in vitro model representing the BMB of our commonly used dairy animals to be used in efflux studies to describe the movement of chemicals/xenobiotics into milk. To date the ideal culture environment for the optimal expression of BCRP and P-gp in pbMEC, to represent expression in the BMB in vivo, is unknown. Further supplementation of the cell culture medium with pregnancy/lactation hormones should be investigated. Next to the expression of BCRP and P-gp in such model, other drug transporters should also be investigated.

Declarations

The authors declare that no funds, grants, or other support were received during the preparation of this manuscript. The authors have no relevant financial or non-financial interests to disclose.

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

This study did not require ethics approval as tissue samples were collected from udder material from an approved abattoir and milk was collected from an approved operating dairy farm.

No consent for participation or publications was required.

Author contributions:

Ronette Gehring and Lerica le Roux-Pullen conceived and designed the study. Lerica le Roux-Pullen, Vienna van de Laarschot and Marjolein Oosterveer-Van der Doelen collected the data. Lerica le Roux-Pullen and Marjolein Oosterveer-Van der Doelen analysed the data. Lerica le Roux-Pullen wrote the main text of the manuscript and prepared the figure. All authors reviewed and approved the final manuscript.

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Figures

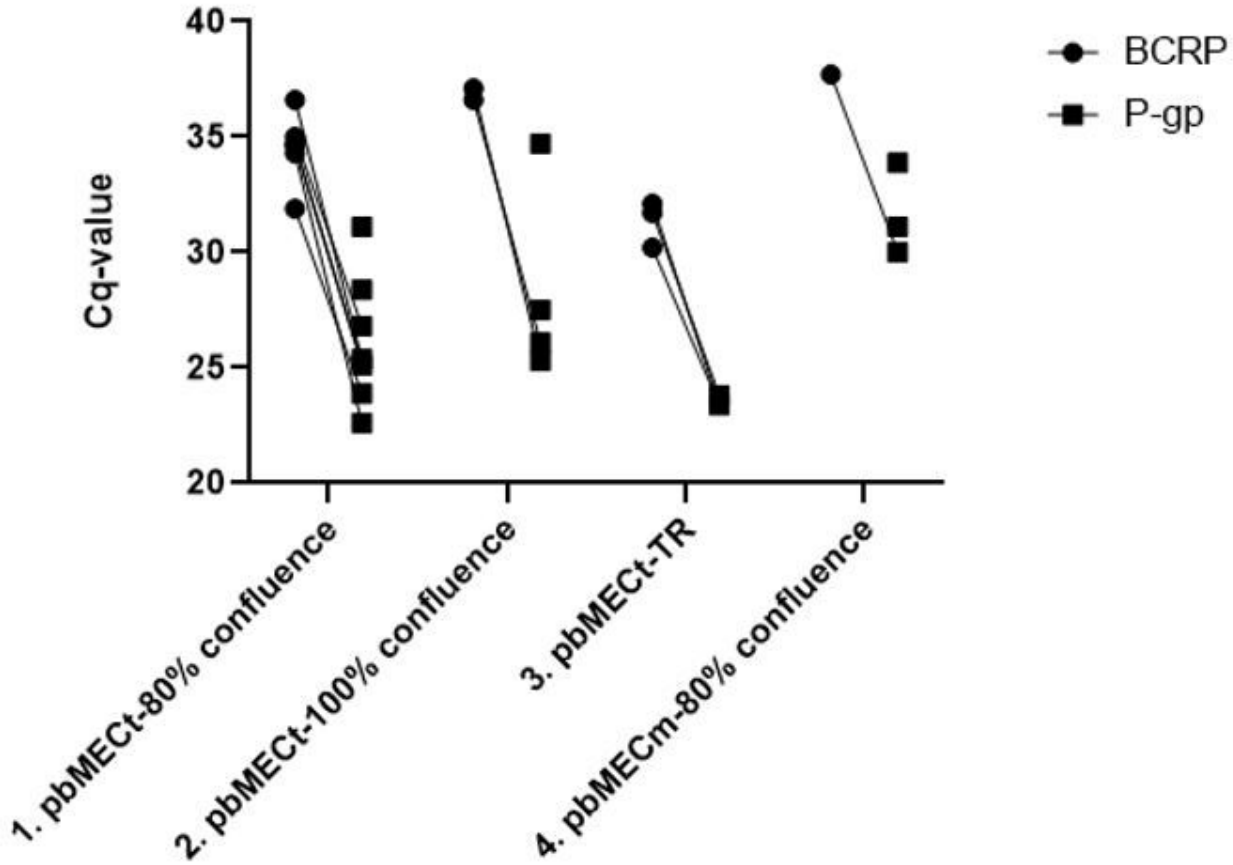

Figure 1

Cq values of mRNA levels of BCRP (circle) and P-gp (square) measured with rt-PCR, grouped per sample. With (1) pbMECt-80% confluence (n=8), (2) pbMECt-100% confluence (n=4), (3) pbMECt-TR (n=3) and (4) pbMECm-80% confluence (n=3).